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Type II Topoisomerase Quinolone Resistance-Determining Regions of *Aeromonas caviae*, *A. hydrophila*, and *A. sobria* Complexes and Mutations Associated with Quinolone Resistance

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Most *Aeromonas* strains isolated from two European rivers were previously found to be resistant to nalidixic acid. In order to elucidate the mechanism of this resistance, 20 strains of *Aeromonas caviae* ($n = 10$), *A. hydrophila* ($n = 5$), and *A. sobria* ($n = 5$) complexes, including 3 reference strains and 17 environmental isolates, were investigated. Fragments of the *gyrA*, *gyrB*, *parC*, and *parE* genes encompassing the quinolone resistance-determining regions (QRDRs) were amplified by PCR and sequenced. Results obtained for the six sensitive strains showed that the GyrA, GyrB, ParC, and ParE QRDR fragments of *Aeromonas* spp. were highly conserved (≥96.1% identity), despite some genetic polymorphism; they were most closely related to those of *Vibrio* spp., *Pseudomonas* spp., and members of the family *Enterobacteriaceae* (72.4 to 97.1% homology). All 14 environmental resistant strains carried a point mutation in the GyrA QRDR at codon 83, leading to the substitution Ser-83→Ile (10 strains) or Ser-83→Arg. In addition, seven strains harbored a mutation in the ParC QRDR either at position 80 (five strains), generating a Ser-80→Ile (three strains) or Ser-80→Arg change, or at position 84, yielding a Glu-84→Lys modification. No amino acid alterations were discovered in the GyrB and ParE QRDRs. Double *gyrA-parC* missense mutations were associated with higher levels of quinolone resistance compared with the levels associated with single *gyrA* mutations. The most resistant strains probably had an additional mechanism(s) of resistance, such as decreased accumulation of the drugs. Our data suggest that, in mesophilic *Aeromonas* spp., as in other gram-negative bacteria, gyrase and topoisomerase IV are the primary and secondary targets for quinolones, respectively.

Mesophilic motile *Aeromonas* is a normal inhabitant of freshwaters and may be considered representative of the riverine autochthonous flora (19). These organisms are also human pathogens and are mainly responsible for gastroenteritis, skin and soft tissue infections, and a variety of clinical syndromes in compromised patients (21); quinolones are the drugs of choice for the treatment of *Aeromonas*-induced infections (22). In a previous study (14), as many as 59% of *Aeromonas* spp. isolated from two European rivers (the Arga River in Spain and the Garonne River in France) were found to be resistant to nalidixic acid; most of them were also resistant to other narrow-spectrum quinolones but remained susceptible to fluoroquinolones. Quinolones are synthetic antibiotics which are normally absent from freshwaters, and quinolone resistance is essentially due to chromosomal mutations (26, 32). Therefore, discharge of quinolones in rivers, probably from an agricultural source, must have selected resistant mutants among indigenous bacterial populations (13). Indeed, quinolones are widely used in veterinary medicine in Europe (30) and more recently in the United States (4); these antibiotics are mostly excreted as unchanged substances and are among the most persistent drugs in the environment (17).

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Mutations that confer quinolone resistance principally alter the target enzymes, the type II bacterial topoisomerases. Both of these enzymes, DNA gyrase and topoisomerase IV, catalyze topological changes in DNA via an ATP-dependent double-strand cleavage and rejoining mechanism. DNA gyrase is primarily engaged in the control of negative supercoiling of DNA, and topoisomerase IV is essentially involved with the decatenation of the interlinked daughter chromosomes (5, 10, 20). Both enzymes are heterotetramers consisting of two types of subunits, GyrA and GyrB in DNA gyrase and the respective homologous proteins ParC and ParE in topoisomerase IV. Gyrase appears to be the primary target for quinolones in gram-negative bacteria since missense mutations in *gyrA* genes are sufficient to render these organisms quinolone resistant (1, 10, 24, 27, 29, 36). In contrast, mutations in the *parC* quinolone resistance-determining region (QRDR) are expressed only in the presence of *gyrA* mutations (10). Virtually all mutations responsible for quinolone resistance have been mapped in a small N-terminal region of the *gyrA* gene, close to the catalytic site Tyr-122, called the QRDR (12, 42). Alterations in the domains of the GyrB and ParE subunits which interact with GyrA and ParC, respectively, can also contribute to quinolone resistance (1, 7, 10, 27, 28, 41, 43).

The aim of the present study was to identify the mutations in the type II topoisomerase genes that confer quinolone resistance in our riverine *Aeromonas* strains. The sequence of the *gyrA* gene of the psychrophilic fish pathogen *A. salmonicida* has been previously established (31), and partial sequences of *A.*

hydrophila gyrB have become available in GenBank since the beginning of the study; but so far, nothing has been reported on the topoisomerase IV-encoding genes in *Aeromonas* spp. Thus, DNA fragments encompassing the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of *A. caviae*, *A. hydrophila*, and *A. sobria* complexes were first determined for reference and environmental susceptible strains and were then compared with those of environmental isolates exhibiting various levels of quinolone resistance.

MATERIALS AND METHODS

Bacterial strains. Of the 20 *Aeromonas* strains examined in this study, 17 (9 *A. caviae*, 4 *A. hydrophila*, and 4 *A. sobria* strains) were selected because they reflected the various antibiotic and quinolone resistance patterns detected among 138 environmental isolates (104 *A. caviae*, 12 *A. hydrophila*, and 22 *A. sobria* strains) previously collected from either the Arga River (Spain) or the Garonne River (France) in 1996 (13, 14). Strains were identified by the criteria of Popoff and Véron (34), as recommended by Holmes et al. (19) for environmental isolates. Briefly, aeroanaerobic gram-negative bacteria with positive oxidase reactions (bioMérieux) were tested for tolerance to 6% NaCl and compound O/129 (Diagnostic Pasteur). The enzymatic activities of ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, and urease, the Voges-Proskauer test, utilization of citrate, indole formation, H_2S production, and carbohydrate fermentation were investigated with the API 20E and API 20NE systems (bioMérieux). The motility was observed on mannitol-motility-nitrate agar (bioMérieux). Hydrolysis of esculin (Merck) and gas formation from glucose metabolism (purple broth base was from BBL; carbohydrate was from Sigma) were also studied. Three type strains of *Aeromonas* were purchased from the Collection de l'Institut Pasteur (CIP) and were included as reference strains: *A. caviae* CIP 7616, *A. hydrophila* CIP 7614, and *A. sobria* CIP 7433. *Escherichia coli* XL1-Blue was used as the recipient in transformation experiments for cloning of the *gyrB* and *parC* gene fragments. All strains were grown on Mueller-Hinton agar.

Antibiotic susceptibility testing. Antibiotic resistance patterns were determined by the disk diffusion method, and MICs were determined by an agar dilution method, according to official guidelines (http://www.sfm.asso.fr). The 10 quinolones tested and their respective manufacturers were as follows: nalidixic acid, Chirex; oxolinic acid, Parke-Davis; pipemidic acid, pefloxacin, and sparfloxacin, Rhône Poulenc Rorer; flumequine, Sigma; norfloxacin, MSD-Chibret; ofloxacin, Roussel Uclaf; and ciprofloxacin and enrofloxacin, Bayer Pharma.

Total DNA extraction. Total cellular DNA was isolated from 3-ml cultures of *Aeromonas* strains grown overnight. Cells were harvested, suspended in TEG buffer (Tris-HCl, 10 mM; EDTA, 1 mM; glucose, 50 mM [pH 8.0]), and lysed with lysozyme (1 mg/ml; 30 min at 37°C) and proteinase K (25 μg/ml; 1 h at 37°C). The DNA was purified by phenol-chloroform extraction, followed by ethanol precipitation.

PCR amplification of QRDRs. PCR amplification of QRDRs was performed with a series of primers, listed in Table 1. Primers AsalgyrAF and AsalgyrAR were designed from the nucleotide sequence of the *A. salmonicida gyrA* gene (31) to amplify the *gyrA* QRDRs of all *Aeromonas* strains. Amplification of the *gyrB* QRDR was carried out first for *A. caviae* 4 with degenerate primers DgyrBF and DgyrBR. After cloning of the QRDR fragment in the PGEM-T vector (see the following section), the PCR product was amplified by using the universal primers (M13 forward and reverse primers) and sequenced. Then, specific primers AcgyrBF and AcgyrBR were designed from this sequence to amplify the *gyrB* QRDRs of all other strains. Similarly, PCR amplification of the *parC* QRDR was performed at first for *A. caviae* 4 by using primers EcparCF and EcparCR, based on the *parC* QRDR of *E. coli* (23). After cloning and sequencing of the PCR product as indicated above, another specific primer, AcparCF, was designed to amplify with primer EcparCR the *parC* QRDRs of all *Aeromonas* isolates. Degenerate primer DparEF and specific primer EcparER, designed from the 3' region of the *E. coli parE* QRDR (23), allowed amplification of the *parE* QRDRs of all *Aeromonas* strains. PCR amplifications were done under standard conditions. After a denaturation step of 5 min at 94°C, amplification was achieved, depending on the primers, over 35 cycles, with each cycle consisting of 1 min at 94°C, 1 min at 50 to 58°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels.

Cloning of PCR products. The products obtained by PCRs with primer pairs DgyrBF-DgyrBR and EcparCF-EcparCR were ligated in the PGEM-T vector with the pGEM-T Vector System cloning kit (Promega), according to the manufacturers' instructions. The ligation mixture was used to transform *E. coli* XL1-Blue by electroporation. Transformed cells were selected on Luria-Bertani agar plates supplemented with ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (0.2 mg/liter), and isopropyl-β-D-thiogalactopyranoside (1 mM). The white colonies were replicated, and the recombinant plasmid DNA was extracted and purified with a Midi kit (Qiagen).

TABLE 1. Primers used to amplify by PCR and to sequence the topoisomerase II fragments of the mesophilic *Aeromonas* spp.

Gene	Primer	Sequence (5' to 3')	Positions[c]
gyrA	AsalgyrAF[a]	TCCTATCTTGATTACGCCATG	58–78
	AsalgyrAR[a]	CATGCCATACCTACCGCGAT	520–539
gyrB	DgyrBF[b]	CCKGGMAARCTKGCRGAVTG	1207–1226
	DgyrBR[b]	RTCYACRTCYGCRTCRGTCAT	1483–1503
	AcgyrBF[a]	CGGAATGCCAGGAGAAAGA	1220–1238
	AcgyrBR[a]	GGTCATGATGATGATGTTG	1470–1488
parC	EcparCF	GAAACCTGTTCAGCGCCGCAT	139–159
	EcparCR[a]	TTCGGTGTAACGCATTGCCGC	371–391
	AcparCF[a]	GTTCAGCGCCGCATCATCTAC	146–166
parE	DparEF [a,b]	GAYGCCTTYATCCTGTGGCTG	1118–1138
	EcparER[a]	GTCCGCATCCGCGAGGATACA	1520–1540

[a] Primers also used for sequencing; F, forward; R, reverse.
[b] Degenerate primers used as universal code.
[c] Numbering as in *E. coli gyrA* (GenBank accession no. X57174), *gyrB* (GenBank accession no. X04341), *parC* (GenBank accession no. M37832), and *parE* (GenBank accession no. M37833) sequences.

DNA sequencing and sequence analysis. PCR amplification products for DNA sequencing were purified with Microspin S-400 HR columns (Amersham Pharmacia Biotech). Automated sequencing of both strands was carried out with an AmpliTaq DNA polymerase FS Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI Prism 377 sequencer (Applied Biosystems Division, Perkin-Elmer), according to the manufacturer's recommendations. To avoid possible misreadings by *Taq* polymerase, three recombinant plasmids originating from different PCR and cloning experiments were selected for sequencing of the products that were obtained by PCR with DgyrBF-DgyrBR and EcparCF-EcparCR and cloned in *E. coli* XL1-Blue. Nucleotide and deduced amino acid sequences were compared by use of Sequence Navigator software (Perkin-Elmer).

Nucleotide sequence accession numbers. The *gyrA*, *gyrB*, *parC*, and *parE* nucleotide sequences of the three reference strains are available in the GenBank nucleotide sequence databases with the following accession numbers: AY027899, AY027902, AF435418, and AF435421, respectively, for *A. caviae* CIP 7616; AY027901, AY027904, AF435419, and AF435422, respectively, for *A. hydrophila* CIP 7614; and AY027900, AY027903, AF435420, and AF435423, respectively, for *A. sobria* CIP 7433.

RESULTS

Antibiotic and quinolone resistance patterns of *Aeromonas* strains. The antibiotic resistance patterns and the MICs of quinolones for the 20 strains of the *A. caviae* ($n = 10$), *A. hydrophila* ($n = 5$), and *A. sobria* ($n = 5$) complexes tested are indicated in Tables 2, 3, and 4, respectively. These isolates included six sensitive strains (one type and one environmental strain for each species), seven strains resistant to a single quinolone (three *A. caviae*, two *A. hydrophila*, and two *A. sobria* strains), and seven strains resistant to multiple drugs (including quinolones) (five *A. caviae* strains, one *A. hydrophila* strain, and one *A. sobria* strain). Nalidixic acid MICs for quinolone-resistant strains of *Aeromonas* were ≥128 mg/liter; most of them remained clinically susceptible to fluoroquinolones, despite a 10- to 4,000-fold increase in the MICs compared with those for susceptible strains.

TABLE 2. Antibiotic resistance patterns, quinolone susceptibilities, and substitutions in the GyrA and ParC QRDRs for strains of the *A. caviae* complex

A. caviae strain	Antibiotic resistance pattern[a]	Quinolone MIC[b] (mg/liter)										Amino acid in QRDRs			
												GyrA		ParC	
		NAL	OA	PI	UB	NOR	PEF	OFX	SPX	CIP	ENR	Position 83	Position 92	Position 80	Position 84
CIP 7616		0.2	≤0.1	≤0.1	0.02	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	0.005	Ser	Met	Ser	Glu
4		≤0.1	≤0.1	≤0.1	≤0.01	0.002	≤0.001	≤0.001	≤0.001	0.002	0.002				
242	Q	128	1	8	2	0.2	0.5	0.2	0.2	0.05	0.1	Arg			
94	Q	128	8	128	16	2	2	1	0.5	0.5	1	Ile			Lys
100	Q	>512	32	256	512	4	16	4	4	1	4	Ile	Leu	Ile	
542	Q, Ctx, (Tc), (C)	128	4	16	4	4	4	1	1	1	0.5	Arg			
33	Q, Ctx, Sxt, KGT	128	8	128	16	4	4	2	2	1	2	Ile		Arg	
2	Q, Tc, (C), (Sxt), T	512	32	64	64	4	2	1	4	0.5	2	Ile		Ile	
495	Q, (Tc), Fos	>512	8	32	8	4	1	0.5	1	0.1	1	Ile			
198	Q, (Tc)	>512	32	128	512	16	16	2	2	4	4	Ile	Leu		Lys

[a] Q, quinolones; Ctx, cefotaxime; Tc, tetracycline; C, chloramphenicol; Sxt, co-trimoxazole; K, kanamycin; G, gentamicin; T, tobramycin; Fos, fosfomycin; parentheses indicate low-level resistance.
[b] NAL, nalidixic acid; OA, oxolinic acid; PI, pipemidic acid; UB, flumequine; NOR, norfloxacin; PEF, pefloxacin; OFX, ofloxacin; SPX, sparfloxacin; CIP, ciprofloxacin; ENR, enrofloxacin.

QRDRs of quinolone-susceptible *Aeromonas* strains. The 441-bp *gyrA* nucleotide sequences (the sizes of the amplified fragments excluding the primers) differed by 49 mismatches, including 12 in the QRDRs (Fig. 1a). The 231-bp *gyrB* sequences varied at 26 positions, 4 of which were located in the QRDRs (Fig. 1b). The 204-bp *parC* sequences of the six mesophilic *Aeromonas* strains exhibited 25 nucleotide changes, including 12 in the QRDRs (Fig. 1c). The 381-bp *parE* sequences showed as many as 69 base differences, although only 5 were situated in the QRDRs (Fig. 1d). These sequences shared 85.4 to 98.7% identity. The levels of similarity between the homologous QRDR fragments of each strain were calculated (data not shown), and the nucleotide variations did not appear to be correlated with the species complex. Analysis of the PCR product generated from *A. caviae* 4 DNA with primer pair DparEF-EcparCR revealed that the *parE* gene was present at less than 80 bp from the 5′ end of the *parC* gene.

The 147 predicted peptide GyrA fragments (amino acids 27 to 173 [*E. coli* numbering]) differed at only four positions outside the QRDR, at positions 50 (Phe or Tyr), 116 (Asn or Ser), 165 (Thr or Asn), and 168 (Val or Ile); compared with *A. salmonicida*, a single change was noted within the QRDR, at position 92 (Met-92→Leu). The 76 peptide GyrB fragments (amino acids 415 to 491) were strictly identical to each other and to seven of the eight homologous sequences of *A. hydrophila* GyrB available in GenBank, with the sequence of the remaining one (GenBank accession no. AF208258) differing at three positions outside the QRDR. The 68 peptide ParC fragments (amino acids 48 to 115) exhibited a single substitution within the QRDR, at position 80 for *A. hydrophila* CIP 7614 (Ser to Ile). The 127 peptide ParE fragments (amino acids 359 to 485) varied at seven positions outside the QRDR: positions 364 (Leu or Met), 367 (Gln or Leu), 368 (Leu or Ile), 371 (Met or Leu), 380 (Met or Leu), 405 (Gly or Asn), and 475 (Asp, Glu, or Asn). The overall homology of the QRDR fragments of mesophilic *Aeromonas* was at least 96.1%. When the most closely related sequences were searched, the QRDR fragments of *Aeromonas* spp. were found to be 72.4 to 97.1% identical to those of *Vibrio* spp., *Pseudomonas aeruginosa*, and *E. coli* but only 37.3 to 74.1% identical to those of *Bacillus subtilis* and *Streptococcus pneumoniae*, which were used as examples of gram-positive organisms.

Mutations in type II topoisomerase QRDRs of quinolone-resistant *Aeromonas* strains. Comparison of the deduced amino acid sequences of the GyrA, GyrB, ParC, and ParE sequences with those of the six sensitive strains described above showed that all quinolone-resistant strains of the *A. caviae* (Table 2), *A. hydrophila* (Table 3), and *A. sobria* (Table 4) complexes carried at least one amino acid substitution in the GyrA QRDR, at position 83: for 10 strains (six *A. caviae*, one

TABLE 3. Antibiotic resistance patterns, quinolone susceptibilities, and mutations in the GyrA and ParC QRDRs for strains of the *A. hydrophila* complex

A. hydrophila strain	Antibiotic resistance pattern[a]	Quinolone MIC[b] (mg/liter)										Amino acid in QRDRs	
		NAL	OA	PI	UB	NOR	PEF	OFX	SPX	CIP	ENR	GyrA position 83	ParC position 80
CIP 7614		0.2	≤0.1	0.5	0.05	0.002	0.002	0.002	0.005	≤0.001	0.005	Ser	Ile
8		≤0.1	≤0.1	0.2	≤0.01	≤0.001	≤0.001	≤0.001	0.002	≤0.001	≤0.001		Ser
256	Q	>512	4	32	4	2	2	2	4	1	2	Arg	Ser
209	Q	256	64	256	512	4	4	2	4	1	2	Ile	
34	Q, Ctx, Tc, (C), Sxt	>512	8	64	32	8	16	4	2	2	2	Arg	Ser

[a] See footnote *a* of Table 2 for definitions of abbreviations.
[b] See footnote *b* of Table 2 for definitions of abbreviations.

TABLE 4. Antibiotic resistance patterns, quinolone susceptibilities, and mutations in the GyrA and ParC QRDRs for strains of the *A. sobria* complex

A. sobria strain	Antibiotic resistance pattern[a]	Quinolone MIC[b] (mg/liter)										Amino acid in QRDRs	
		NAL	OA	PI	UB	NOR	PEF	OFX	SPX	CIP	ENR	GyrA position 83	ParC position 80
CIP 7433		≤0.1	≤0.1	0.2	0.02	0.002	≤0.001	≤0.001	0.005	≤0.001	≤0.001	Ser	Ser
208		≤0.1	≤0.1	0.5	≤0.01	0.002	≤0.001	≤0.001	0.002	≤0.001	≤0.001		
536	Q	256	1	8	4	0.5	0.5	0.2	0.2	0.1	0.2	Ile	
367	Q	128	8	128	512	2	2	0.5	0.5	0.5	1	Ile	Arg
384	Q, Tc, Sxt	128	4	32	32	4	2	1	1	1	1	Ile	

[a] See footnote *a* of Table 2 for definitions of abbreviations.
[b] See footnote *b* of Table 2 for definitions of abbreviations.

A. hydrophila, and three *A. sobria* strains) a GC-to-TT mutation at nucleotide positions 248 and 249 resulted in a Ser-to-Ile substitution; in 4 other mutants (two *A. caviae* and and two *A. hydrophila* strains), a A-to-C mutation at nucleotide position 247 led to a Ser-to-Arg substitution. In two *A. caviae* strains, Met-92 was replaced by Leu, as in the *gyrA* QRDR of *A. salmonicida*. In addition, seven strains (five *A. caviae* strains, one *A. hydrophila* strain, and one *A. sobria* strain) exhibited amino acid modifications in the QRDR of ParC, either at position 80 or at position 84: in five strains, a A-to-C transversion at position 263 or a G-to-T transversion at nucleotide position 264 gave rise to a substitution of Ser-80 to Arg or Ile, respectively; in two strains, a G-to-A transition at nucleotide position 275 yielded a replacement of Glu-84 by Lys. Multiple amino acid variations were found outside the QRDRs of GyrA and ParE at the same positions and with the same residues as in quinolone-susceptible strains of *Aeromonas*, except that Leu was always present at position 364 and Ile rather than Met was found at position 371 of ParE (data not shown). No amino acid changes were found in the GyrB sequences of the 14 quinolone-resistant strains of mesophilic *Aeromonas* spp.

Among the *A. caviae* isolates tested (Table 2), strain 100, which carried a mutation in both the GyrA (Ser-83→Ile) and the ParC (Ser-80→Ile) QRDRs, was more resistant to all quinolones tested than strain 94, which differed by a mutation in the ParC QRDR (Glu-84→Lys). The MICs of all drugs were higher for strain 94 than for strain 242, which carried a single mutation in the GyrA QRDR (Ser-83→Arg). Strain 542, which presented with an associated low level of resistance to tetracycline and chloramphenicol, was 5 to 20 times more resistant to fluoroquinolones than strain 242, despite an identical substitution in the GyrA QRDR (Ser-83→Arg). Similarly, among the *A. hydrophila* strains tested (Table 3), strain 209, which had a double mutation (Ser-83→Ile in GyrA, Ser-80→Ile in ParC), was 8 to 128 times more resistant to oxolinic acid, pipemidic acid, and flumequine than strain 256, which had a single mutation, Ah256 (Ser-83→Ile in GyrA). The MICs of most quinolones were higher for strain 34 than strain 256, although both strains carried the same mutation in GyrA (Ser-83→Arg), but the former strain was multidrug resistant, with resistance to chloramphenicol at low levels. Likewise, among the *A. sobria* isolates tested (Table 4), strain 536 (which had Ser-83→Ile in GyrA) was less resistant than strain 367 (which had Ser-83→Ile in GyrA and Ser-80→Arg in ParC) and strain 384 (which had Ser-83→Ile in GyrA and which was multidrug resistant).

DISCUSSION

Aeromonas spp. are exquisitely susceptible to quinolones (39), and until now they have been found to be consistently susceptible to these antibiotics in most parts of the world (22). However, the first nalidixic acid-resistant strain of *A. hydrophila* was reported in 1987 among clinical isolates from Asia (8), and in 1996, 4% of the clinical mesophilic strains of *Aeromonas* isolated in Taiwan were resistant to more than 2 mg of pefloxacin per liter (25). The mechanism of this resistance has not been investigated. We have found a very elevated numbers of quinolone-resistant strains among mesophilic *Aeromonas* strains isolated from two European rivers (13, 14). Such resistance is of clinical concern, since freshwaters are the main source of *Aeromonas*-induced infections in humans. Because mutations in the type II topoisomerase genes are the principal mechanisms of quinolone resistance, the sequences of the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of three mesophilic *Aeromonas* species were determined, and mutations associated with quinolone resistance were investigated.

The nucleotide sequences of the *gyrA*, *gyrB*, *parC*, and *parE* QRDR fragments of susceptible *A. caviae*, *A. hydrophila*, and *A. sobria* strains exhibited some genetic heterogeneity. In contrast, Oppegaard and Sørum (30) have reported a remarkable degree of nucleotide sequence identity among *gyrA* fragments from different strains of *A. salmonicida*. These strain-to-strain variations might be related to taxonomic uncertainties or gene polymorphism, or both. Indeed, the taxonomy of mesophilic *Aeromonas* strains has undergone deep revisions in the last two decades. While the psychrophilic aeromonads represent a homogeneous collection of strains, mesophiles are a heterogeneous cluster and have been allocated to 13 genomic species by DNA-DNA hybridizations (21). Biochemical identification is considered >85% accurate to the phenospecies level, e.g., to the *A. hydrophila*, *A. caviae*, and *A. veronii* ("*A. sobria*") complex levels (21). Analysis of the restriction fragment length polymorphisms of PCR-amplified fragments of the 16S rRNA gene (6, 15) did not allow further specification of this identification (data not shown), and thus, strains were finally referred to as "complexes" rather than "species." On the other hand, substantial polymorphism of the topoisomerases genes, including the QRDR sequences, has previously been described in a number of organisms (1, 9, 27, 29, 38). Surprisingly, the *parC* and *parE* genes were found to be contiguous on the chromosome of the mesophilic *Aeromonas* strains, whereas this feature is mainly observed in gram-positive organisms (10, 20).

a

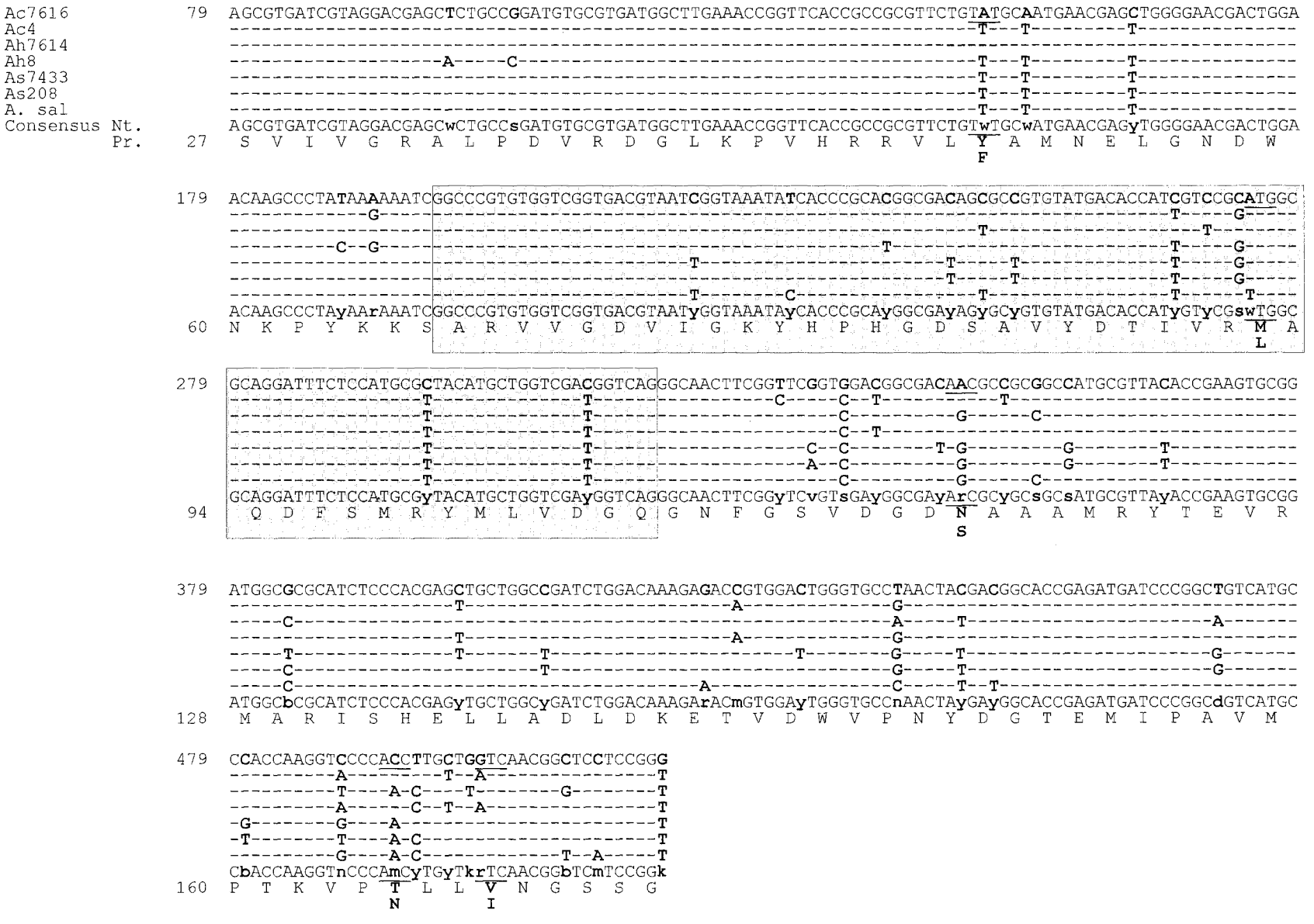
Ac7616
Ac4
Ah7614
Ah8
As7433
As208
A. sal
Consensus Nt.
Pr.
79
27
179
60
279
94
379
128
479
160

FIG. 1.

b

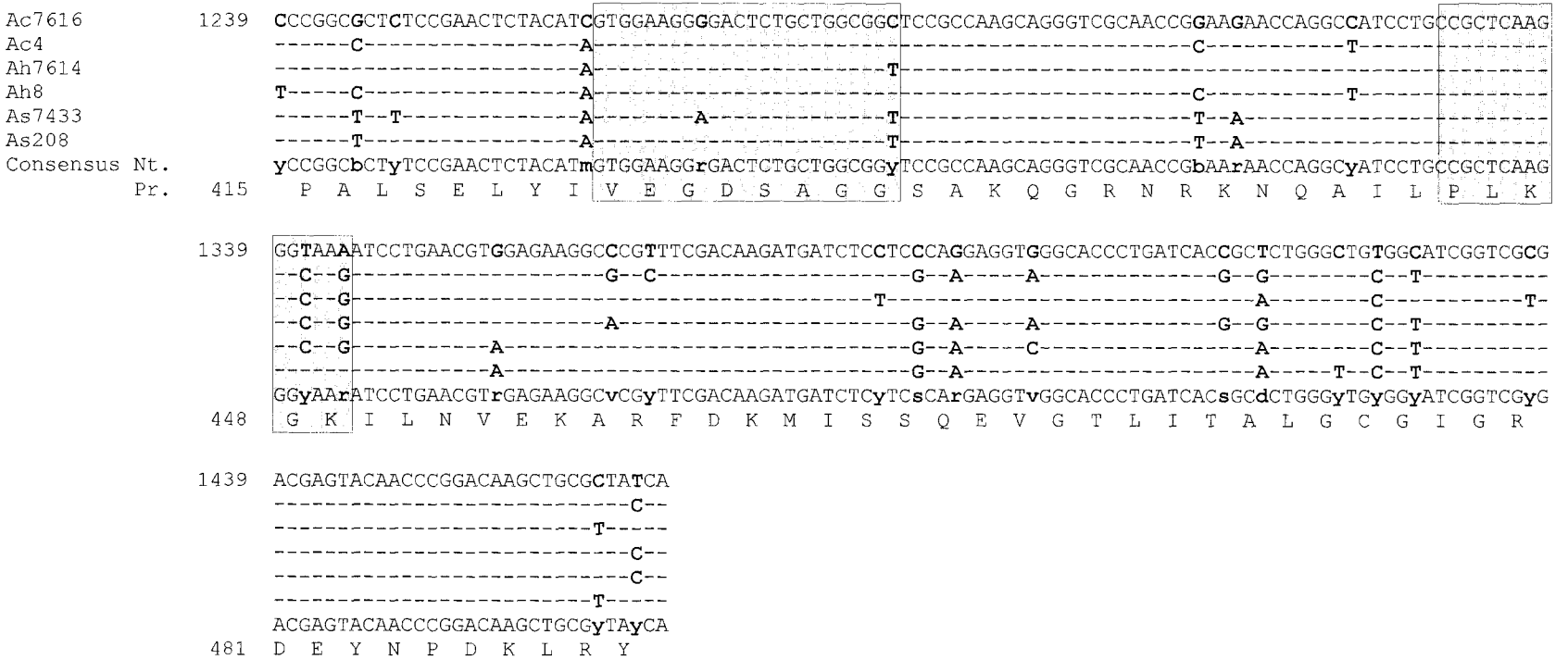

FIG. 1—*Continued.*

c

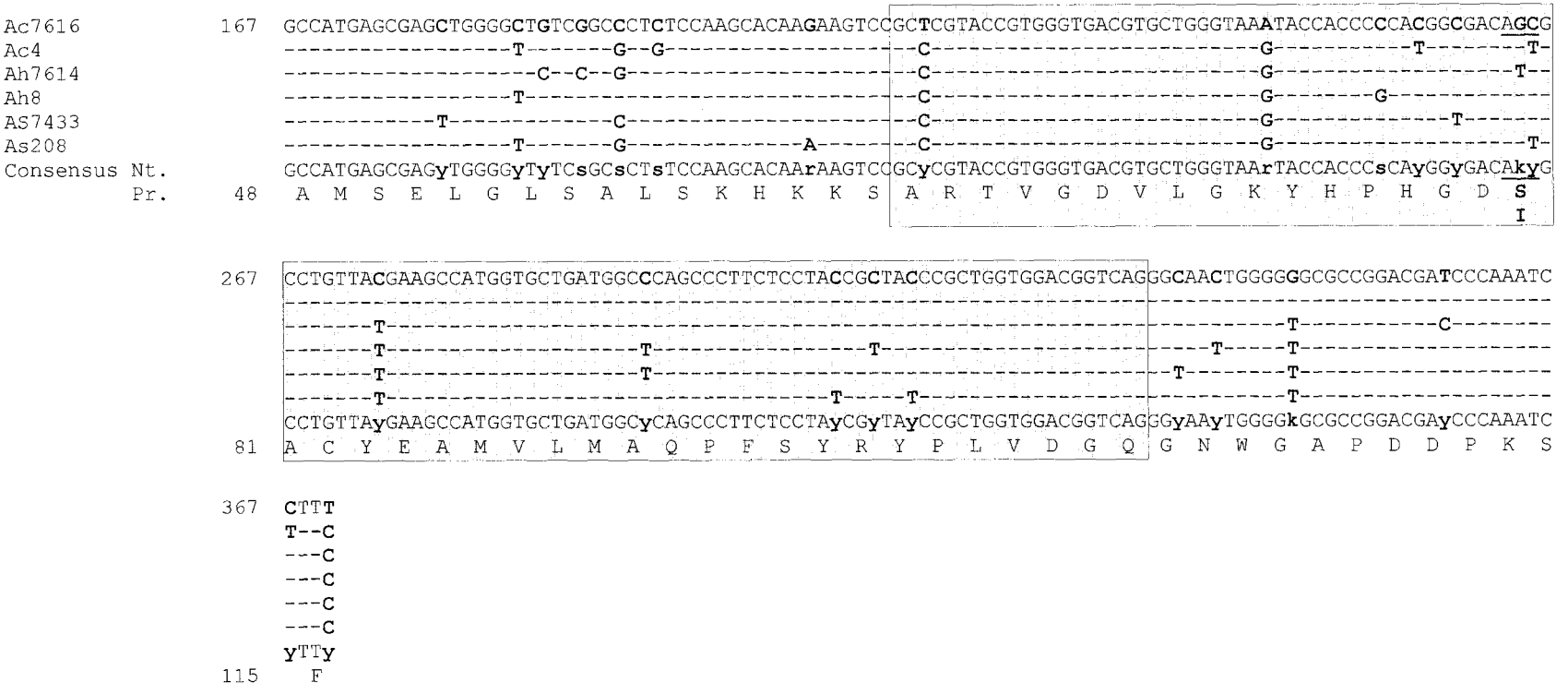

FIG. 1—*Continued*.

d

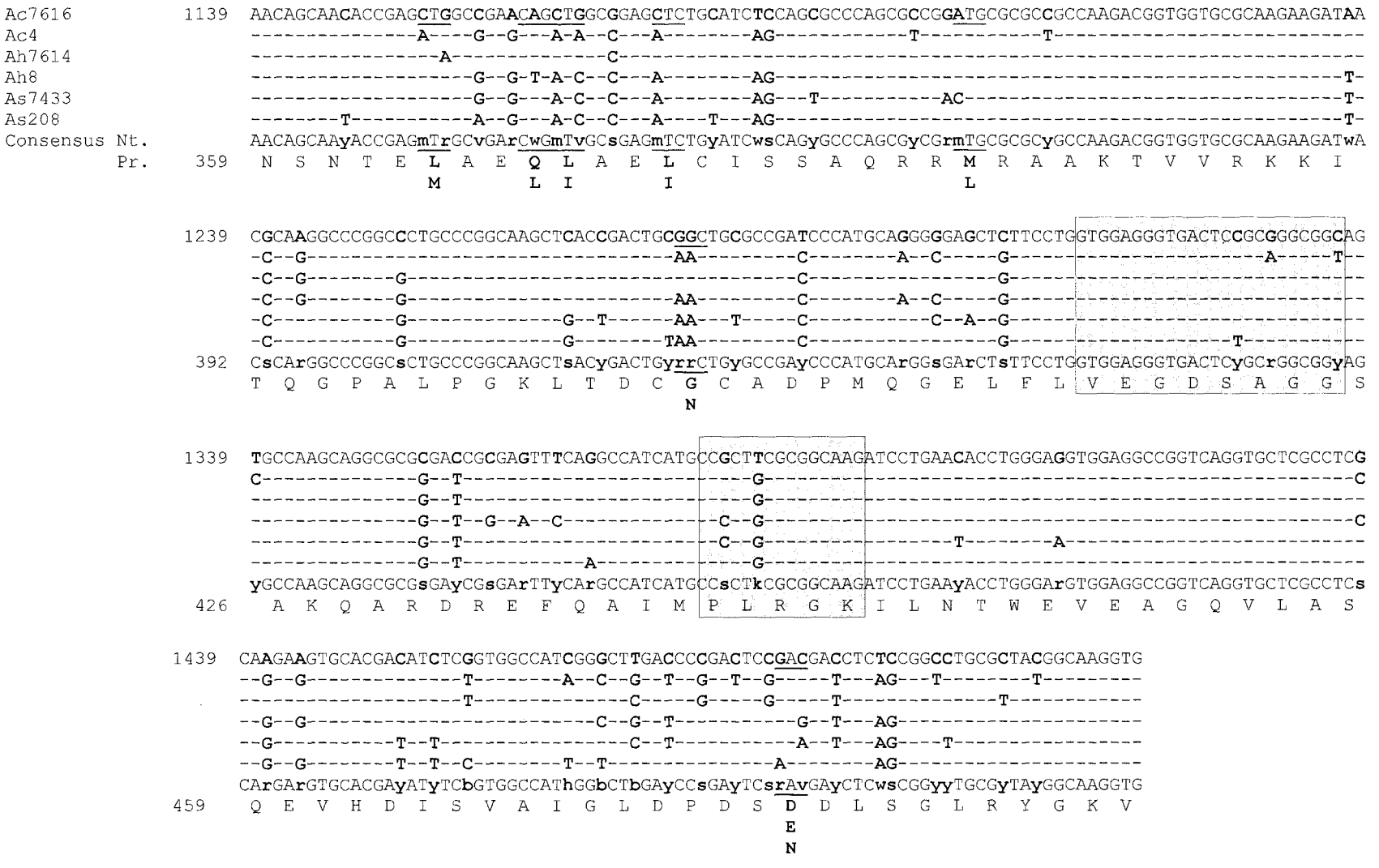

FIG. 1. Nucleotide sequences of *gyrA* (a), *gyrB* (b), *parC* (c), and *parE* (d) fragments containing the QRDRs of *Aeromonas* spp. The sequences of the six sensitive strains of *A. caviae* (Ac), *A. hydrophila* (Ah), and *A. sobria* (As) are as determined in the present study; Ac7616, Ah7614, and As7433 are the reference CIP strains. The *A. salmonicida* (A. sal) *gyrA* sequence is as reported by Oppegaard and Sørum (30). The *E. coli* numbering system is used (GenBank accession numbers are given in footnote *c* of Table 1). QRDRs are framed and shaded. Nucleotide differences are indicated with boldface characters and are underlined when the difference led to amino acid changes. The corresponding nucleotide (Nt.) and proteic (Pr.) consensus sequences are shown.

The deduced amino acid sequences of the GyrA, GyrB, ParC, and ParE fragments of the *A. caviae*, *A. hydrophila*, and *A. sobria* complexes were highly homologous to each other; mostly similar to those of *Vibrio* spp., *Pseudomonas* spp., and members of the family *Enterobacteriaceae*; and more distant from those of gram-positive organisms, in agreement with the phylogenetic relationships of these bacteria. Within the GyrA QRDRs of *Aeromonas* spp. (30, 31), a serine residue was present at position 83, as in *Vibrio parahaemolyticus* (29) and most members of the family *Enterobacteriaceae* (38), whereas a threonine is present at position 83 in *P. aeruginosa* and some other organisms (10, 20). The GyrA QRDRs of mesophilic *Aeromonas* strains differed from that of *A. salmonicida* by a single amino acid change, Met-92→Leu, as, among members of the family *Enterobacteriaceae*, is found in *Providencia stuartii* (38). Multiple variations were found outside the QRDRs of GyrA (amino acids 50, 116, 165, and 168) and ParE (amino acids 364, 368, 371, 380, 405, and 475) and involved like residues that are also present in the most closely related species.

All highly quinolone-resistant strains of mesophilic *Aeromonas* examined in the present study carried an amino acid change in the GyrA subunit, at position 83. This observation strongly suggests that in mesophilic *Aeromonas* strains, as in other gram-negative bacteria, DNA gyrase is the primary target of quinolones (32). All mutations in *gyrA* responsible for high-level quinolone resistance are clustered within the QRDR (amino acids 67 to 106) (42), and those that alter residue 83 are both the most frequently encountered and those that confer the most significant increase in the level of quinolone resistance, followed by substitutions of amino acid 87 (1, 2, 10, 27, 42). For 10 strains of mesophilic *Aeromonas* the substitution was Ser-83→Ile, and for 4 strains the substitution was Ser-83→Arg. Quinolone resistance-determining mutations at positions 83 and 87 are always to hydrophobic amino acids, but the type of allele varies widely (1, 10, 11, 27, 29, 38, 42); in *A. salmonicida*, all quinolone-resistant strains investigated so far carried a Ser-83→Ile substitution (30). Double missense mutations in the *gyrA* QRDR, particularly at both position 83 and position 87, have been associated with an increase in the level of resistance to fluoroquinolones (1, 2, 11, 27, 32, 37). However, other amino acid modifications found in the GyrA fragments of quinolone-resistant *Aeromonas* strains have previously been identified in wild-type strains of *Aeromonas* and/or in other quinolone-susceptible species and, therefore, are unlikely to result in decreased quinolone susceptibility. Rarely, quinolone resistance-determining mutations have been mapped in GyrB, at Asp-426 and Lys-447 in *E. coli* (10, 37, 41) or at Ser-464 in *P. aeruginosa* (1, 27). Nevertheless, no substitutions were discovered in the GyrB sequences of our quinolone-resistant strains of *Aeromonas*.

In fact, among highly fluoroquinolone-resistant strains, mutants with double *gyrA-parC* mutations occur more frequently than those with double *gyrA-gyrB* mutations (1, 10, 24, 27, 29, 36). Actually, alterations in the ParC QRDR (amino acids 64 to 103) were detected in 7 of the 14 quinolone-resistant strains investigated. For five strains, the substitution was at position 80, either Ser-to-Ile (three strains) or Ser-to-Arg; for two strains, the substitution was Glu-84 to Lys. Amino acids 80 and 84 in ParC are homologous to residues 83 and 87 in GyrA (10). Substitution to hydrophobic and positively charged amino acids at these codons, respectively, are both the most common and those that convey the highest levels of quinolone resistance (1, 18, 24, 27, 29, 36). Accordingly, a mutation at position 80 conferred more resistance than a mutation at position 84. For *Aeromonas* strains carrying a double *gyrA-parC* mutation, quinolone MICs were higher than those for strains with a single *gyrA* mutation (1, 2, 10, 24, 27). No resistant mutants with a *parC* mutation alone were observed. Moreover, wild-type strain *A. hydrophila* CIP 7614 carried a substitution of Ser-80 to Ile in the ParC QRDR. These data support the view that topoisomerase IV is a secondary target for quinolones in *Aeromonas* spp. (10, 24). Exceptionally, quinolone resistance-determining mutations in *parE* have been characterized in gram-negative organisms: at Leu-445 in *E. coli* (7, 11, 35) or at Asp-420 in *P. aeruginosa* (1). However, no substitutions were detected within the ParE QRDRs of our quinolone-resistant strains of *Aeromonas*, and the amino acid variations found outside the QRDRs have already been recognized in quinolone-susceptible strains or species.

Finally, reduced levels of uptake or an active efflux system(s) (33) might explain why some multidrug-resistant strains of *Aeromonas* were more resistant to fluoroquinolones than other strains were, despite identical target modifications. Indeed, many of our riverine isolates exhibited low levels of resistance to tetracycline and/or chloramphenicol, and the resistance was not transferable (13). Low levels of resistance to multiple antibiotics, including quinolones, tetracyclines, and chloramphenicol, have been found to be associated with changes in outer membrane protein profiles and have been ascribed to decreased permeability in *A. hydrophila* (3, 16, 40).

In conclusion, the GyrA, GyrB, ParC, and ParE QRDRs of the *A. caviae*, *A. hydrophila*, and *A. sobria* complexes were highly similar or identical, despite some degree of genetic heterogeneity. Quinolone resistance was primarily related to mutations in the *gyrA* gene since all quinolone-resistant strains carried a substitution of the Ser at position 83, with a mutation to Ile being more frequent than that to Arg. The presence of an additional mutation in ParC, either a Ser-80→Ile or Arg change or a Glu-83→Lys change, was demonstrated in seven strains for which quinolone MICs were higher. Additional mechanisms such as decreased levels of drug accumulation probably account for the highest levels of quinolone resistance.

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